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Signed this 16th day of August 2004



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For and on behalf of RWS Group Ltd

Title:

**CONJUGATED LINOLEIC ACID AND TRIGLYCERIDE, NEW METHODS  
OF SYNTHESIS AND USE**

5

Abstract:

The invention relates to new methods of production of  
conjugated linoleic acid (CLA) in the form of free  
fatty acid or of triglyceride of high configurational  
10 and/or chemical purity. Moreover, new methods of  
topical and oral treatment are contemplated by means of  
dietary preparations and cosmetic compositions capable  
of optimizing the action of said multiactive metabolic  
lipid.

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DESCRIPTION

Appended to the patent application for an INDUSTRIAL INVENTION with the title:

**CONJUGATED LINOLEIC ACID AND TRIGLYCERIDE, NEW METHODS OF SYNTHESIS AND USE**

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DESCRIPTION

The invention relates to the synthesis of free conjugated linoleic acid (CLA) by alkaline isomerization of vegetable oils in a glyceric medium, as well as CLA obtained from grapeseed oil, as well as the synthesis of CLA triglyceride from castor oil by elimination of a 12-alkyl- or -aryl-sulphonic group previously formed.

The invention further relates to methods of topical and oral treatment by means of dietetic preparations and cosmetic compositions based on free CLA or triglyceride.

The designation CLA comprises a mixture of geometrical and positional isomers of linoleic acid. CLA occurs in foods of animal origin, in particular in bovine meat and in dairy products, though in extremely limited amounts such as to make their extraction-purification from natural sources impracticable for commercial purposes.

The commercially available CLA is generally produced by alkaline isomerization of vegetable oils at high temperature in the presence of a solvent, for example diethyleneglycol, ethylene glycol or propylene glycol.

Typically, said CLA is formed to about 60-70% from c9,t11-, t10,c12-, t9,t11- and t10,t12- octadienoic acids; to 5-7% from the c9,c11- t9,c11-, c10,c12-, and c10,t12 isomers; and to 28-33% from monounsaturated

fatty acids (oleic, elaidic and palmitoleic acid) and saturated fatty acids (myristic, palmitic and stearic acid) initially present.

At the end of the operation, water is added to the  
5 reaction mixture, it is neutralized with inorganic acid, then extracted with ether solvent, followed by phase separation and solvent distillation.

However, the product can still contain appreciable amounts of the reaction solvent (e.g. diethyleneglycol)  
10 as well as residual traces of ether or hexane, therefore it can be characterized by the presence of impurities.

We have developed a method of alkaline isomerization characterized by the use of glycerol,  
15 i.e. which aims to avoid the drawbacks mentioned above, to produce a CLA for topical and oral use.

In the method of the present invention, a vegetable oil with high content of linoleic acid and the glycerol are loaded in a reactor in the weight  
20 ratio 0.5:1 to 4:1 (from 1 to 5 equivalents of fatty acids), it is made inert with nitrogen or argon, heated to temperatures from 200 to 240°C, preferably near the top of this range, for times varying from 2 to 6 hours, depending on the temperature. At 230°C a reaction time  
25 of about 2.5 hours will be sufficient, to obtain a degree of conversion of the linoleic acid to CLA greater than 98%.

This is followed by cooling, with increase in viscosity by the CLA soaps, and at about 90°C a volume  
30 of water is introduced, heating to remove the monoglycerides that are present (approx. 5%).

Then the whole is neutralized with sulphuric acid or HCl (between 10 and 50% w/v), leaving the phases to separate for 6-12 h, until the fatty acids separate as  
35 the upper phase, which are then washed with water, or treated in a stream of steam, and optionally dried, filtered and centrifuged, deodorized and/or clarified by known methods.

The linoleic acid-CLA conversion yields of the method of the present invention are of the order of 70-80% at 200°C for 4-5 h, and up to 98% at 230°C in 3-4 h. At temperatures above 235°C there may be the start of reactions of decomposition or dimerization, whereas at 245°C the formation of acrolein is observed.

The raw materials preferably used in the present method are vegetable oils with high content of linoleic acid, such as sunflower oil, Carthamus oil or corn oil, in the form of glycerides or other esters, free fatty acids, or their mixtures.

Surprisingly, a CLA obtained from grapeseed oil was not known, prior to the present invention.

Grapeseed oil has the highest content of linoleic acid, 72-76%, higher than Carthamus oil (70-72%), sunflower oil (60-62%), and corn oil (approx. 52%), against the minimum content of linolenic acid, isomerization of which leads to highly unsaturated conjugated products of doubtful metabolic value.

A further object of the invention is therefore CLA for topical and oral use obtained from grapeseed oil by alkaline isomerization in the presence of any solvent with a boiling point above 240°C.

A CLA that is particularly preferred for our purposes can be obtained from ricinoleic acid, giving a CLA of high chemical and configurational purity, with a composition that does not differ substantially from that found in biological tissues.

An example of a method for the preparation of a relatively pure CLA is described by Berdeaux et al., in JAOCS, 74, 8, 1011-1015, 1997, and envisages elimination of the sulphonates of methyl ricinoleate to give a mixture composed primarily of c9,t11- c9,c11- c9,t12-octadienoic acids in relative proportions of 10:3:1.

In some topical or oral applications it would be advisable to have the triglyceride of CLA, eliminating the drawback of the acidity due to CLA in free form.

The triglyceride of CLA can, in principle, be produced by known methods, such as the transesterification of a glyceric ester, for example by the action of metallic sodium on a mixture composed of triacetin and the CLA methyl ester.

For convenient, direct production of a triglyceride of CLA at high purity for topical and oral use, we have developed an efficient industrial method, comprising the formation of the 12-tosylate of ricinus triglyceride, selectively converted by tertiary amines to triglyceride at 90% of CLA, containing exclusively the c9,t11-, and c9,c11-octadienoic acids in proportions from 4:1 to 5:1.

A further object of the present invention is therefore a triglyceride of CLA with high obtained from castor oil by elimination of a previously formed 12-alkyl or 12-aryl sulphonic group.

CLA was initially studied with particular interest for the ability to inhibit breast cancer and prostate cancer, by mechanisms that were not entirely clear, attributed in part to the ability to modulate the activity of lymphocytes and macrophages.

After attracting attention for its antineoplastic properties, CLA has recently been at the centre of attention for various positive biological effects on higher organisms, for example anti-atherogenic, anti-hyperinsulinaemic and slimming action.

Research of an academic nature is in this case superimposed on a series of patents claiming the dietary-curative use of CLA.

However, we have discovered that the oral administration of dietetic products based on CLA leads to an increase in peroxidative stress, with increase of the known risks connected with the formation of lipoperoxy radicals.

This is reflected in the fact that CLA is a reactive molecule, able to combine easily with oxygen and produce the peroxide radical ( $\text{LOO}^\circ$ ), then transformed to hydroperoxide ( $\text{LOOH}$ ), successively

producing  $L^\circ$  (chain propagation) and a cyclic peroxide. The cyclic peroxide finally tends to isomerize to cyclic endoperoxide and the latter reacts with the singlet oxygen ( $O_2^\circ$ ), to form malondialdehyde and other  
5 oxygenated lipid fragments, which are able to propagate the lipoperoxidative chain.

We have discovered that this lipo-oxidative effect is inhibited by the concomitant presence of sufficient amounts of antioxidants in the organism.

10 The present invention therefore relates further to a method for the oral administration of CLA in combination with dietetically acceptable antioxidants.

The method, capable of providing the typical benefits of the metabolic lipid in question (CLA),  
15 comprises the administration of a dietetic preparation containing:

- i) CLA in the form of free acid or triglyceride, in amounts between 0.01 and 50 wt.% of the preparation.
- 20 ii) Dietetically acceptable antioxidants, in amounts such that the weight ratio of antioxidant to CLA is between 1:2 and 5:1.
- iii) Dietetically acceptable nutrients and excipients, for the remainder of the preparation.

25 The antioxidants suitable for our purpose include, but are not limited to, the antioxidants contained in the seeds, the leaves, the flowers and the bark or cortex of fruit and vegetables, and can be extracted from natural sources or can be the equivalents of  
30 synthetic origin.

Representative antioxidants are some amino acids (e.g. histidine and valine), and flavonoids (e.g. aromadendrene, morin, quercetin, hesperetin, naringenin, kaempferol, apigenine, luteolin, fisetin,  
35 fustin, rutin, myricetin), the anthocyanins (e.g. pelargonidin, cyanidin, delphinidin), the catechols (e.g. catechol, epicatechol, gallic acid, gallic acid, proanthocyanosides), the carotenoids and retinoids (isomers of carotene, lycopene, zeaxanthin, retinol,

cathaxanthin), and tocopherols (e.g. alpha-, beta- and gamma-tocopherol), and tannins (e.g. gallic acid, propyl gallate and gallic esters, tannic acid), and phospholipids (e.g. egg and soya lecithin), L-ascorbic  
5 acid and esters, alpha-hydroxyacids (e.g. lactic acid, citric acid, L-tartaric acid), the polyketides of Monascus red, substances from peanut and rice (e.g. cardanol, cardol, anacardic acid, oryzanol, anacardic oil), thiodipropionic acid and laurylic ester (DLTBP),  
10 alpha-lipoic acid, nordihydroguaiaretic acid (NDGA), guaiacol, hydroxytyrosol and esters from olive oil (e.g. oleuropeine, verbascoside), other phenols from spices and herbs (e.g. rosemary, cloves, peppers, cinnamon, ginger, paprika (TBHQ), citrus oils,  
15 trihydroxybutyrophenol (THBP), BHT and BHA), maclurin, ethoxyquine, ellagic acid, musizine, boldine, as well as their corresponding glycosides, esters and ethers, and their combinations.

The dietetic preparations based on CLA and natural  
20 antioxidants can be used as dietary supplements or pharmaceutical preparations, preferably being administered in dosed forms, such as tablets and capsules.

Said dietetic preparations can be in the form of  
25 emulsion or preparation for the sportsman, baby-food, milk-dairy product, alcoholic solution, or other formulation for human nutrition.

The dietetic preparations are administered by mouth, preferably at a dosage from 10 to 50 mg of CLA  
30 per kg of body weight.

The optimum daily dosage of the compositions depends on body weight, so as to supply the subject with the amount of CLA necessary for the prophylactic treatment of forms of atherosclerosis, diabetic and  
35 oncologic treatments, or treatment for sport, immunostimulant and slimming typical of the consumption of CLA.

The compositions are preferably administered 1-3 times a day in tablets or capsules containing 500-1000



mg of free CLA or triglyceride and 250-5000 mg of natural antioxidants, preferably avoiding taking total doses greater than 3000 mg/CLA per day.

To date, the dermatologic application of CLA is limited to the zinc salts, as described in patent PCT-09817269, concerning the preparation of compositions for use in the treatment of skin disorders: eczema, forms of psoriasis and dermatitis.

We found surprisingly that the topical effects of using CLA lead to a significant improvement in cellulitic condition.

The phenomenon of "bumpiness" of the skin, of the thighs and buttocks is commonly termed "cellulite", a dermohypodermosis with oedemato-sclerotic panniculopathy in which the fibroblastic reaction prevails over vascular exchanges. As noted by Rosenbaum M. et al. (Surg. of Plast. Reconstr., 101(7):1934-9 1998), biopsies and sonography *in vivo* of the cellulitic thigh show diffusion of adipose tissue in the dermatic reticulum.

We have now found that the topical application of CLA speeds up the reduction of fatty deposits localized in the cellulitic areas, therefore giving significant advantages in the treatment of the aesthetic defect.

Therefore a further object of the present invention is a method of treatment of the cellulitic condition by means of cosmetic compositions containing free CLA or triglyceride.

Trials conducted on women with healthy dermatologic conditions, but with areas with cellulitic diffusion, did not show any signs of irritation or intolerance, and CLA is moreover particularly indicated on account of its physicochemical properties: it has high cutaneous compatibility, it is completely soluble in the oily phase of cosmetic compositions, and it has high capacity for absorption (penetration) through the horny layer.

The anti-cellulitic activity seems to be due to a reduction of adipose deposition and increased lipolysis

in the adipocytes. A mechanism of involvement in lipid storage is postulated, in which CLA presumably causes the mobilization of the lipid reserves, by increasing lipase-sensitive hormonal activity and by inhibiting lipase lipoproteins.

The hypothesis of an action of inhibition of the proliferative phase of the adipocytes, as would follow from the experiments conducted by Satory D.L. and Smith S.B. (J. Nutr., 82, 1, 92-7, 1999) and by Brodie A.E. et al. (J. Nutr., 129, 3, 602-6, 1999), seems to us to be more plausible.

Furthermore, as it has been found that phenomena of an inflammatory type contribute to the phenomenon of cellulite formation, the known anti-PGE properties that CLA possesses would be effectively active in said mechanism.

The present invention can provide a method of intensive treatment of cellulite based on CLA and/or the corresponding triglyceride, which can be used as such, at concentrations from 60 to 90 wt.%.

Cosmetic use may, however, benefit from other ingredients that can improve the appearance of the cosmetic zones, facilitate the penetration of CLA and/or increase the efficacy of the anti-cellulitic treatment. Another object of the invention therefore comprises cosmetic compositions that contain CLA in the form of free fatty acid or triglyceride, with a content between 0.5 and 50 wt.%, preferably between 1 and 5 wt.%.

In a preferred embodiment of the present invention, CLA is used in combination with other anti-cellulitic substances.

In view of the aetiological uncertainty, cellulitic areas are treated with a variety of active ingredients having various mechanisms. The combinations with other anti-cellulite substances include those mentioned by the authors Di Salvo in "Controlling the Appearance of Cellulite: AHAs and Cellulite Products" (C & T Ingredient Resource Series, 21-27) and Curri in

"Local Lipodystrophy and Districtual Microcirculation" (Cosmetics and Toiletries, 109, 51-53, September 1994), cited here as reference.

Particularly preferred examples of anti-cellulitics are xanthins, especially theophylline and caffeine on account of their excellent availability and efficacy, for example present from 0.5 to 3.0 wt.% of the composition, to maximize efficacy and cost.

Other examples of anti-cellulitics are stimulators of collagen synthesis, for example betulinic acid, ascorbates, the triterpenoids of *Centella asiatica*, inositol phosphate and phytic acid, vasodilators - which improve the sparse vascularity associated with cellulitic areas - for example ginsenosides, ivy, minoxidil, methylnicotinate, as well as salicylate, alpha2-adrenergic antagonists and beta-adrenergic agonists.

Other examples of anti-cellulitics are other lipolytic agents, which may act by synergistic mechanisms with that of CLA in the mobilization of the adipose deposits, for example extracts from *Polygala tennifolia*, from *Platycodon grandiflorum*, from *Hibiscus abelmoschus*, from *Kochia scoparia*, etc.

Another preferred combination of CLA in the present invention comprises alpha hydroxy acids, preferably monocarboxylic acids, for example lactic acid, glycolic acid, mandelic acid, and their mixtures, for increasing percutaneous absorption. The amount of alpha hydroxy acids contained in the composition according to the present invention varies from 1.5 to 15 wt.%, more preferably from 3 to 12 wt.%.

Other types of active ingredients are considered in the present invention. Although not limited to these categories, general examples include anti-wrinkle and anti-inflammatory ingredients and skin depigmentation ingredients.

Examples of anti-inflammatory ingredients are glycyrrhizinic derivatives, flavonoids, alpha-bisabolol, rosmarinic acid, azulene, asiaticoside,

ruscogenin, aescine, betulinic acid and their derivatives. Examples of depigmentation ingredients are hydroquinone, arbutin, and kojic acid. Examples of anti-wrinkle ingredients are retinol and its derivatives, tocopherol and its derivatives, the salicylates and derivatives.

Another preferred optional ingredient is selected from the essential fatty acids - for promoting epidermal lipid biosynthesis and supplying the lipids of the epidermal barrier - preferably selected from linoleic acid, gamma-linolenic acid, homo-gamma-linolenic acid, columbinic acid, eicosa-(n-6,9,13)-trienoic acid, arachidonic acid, gamma-linolenic timnodonic acid, hexaenoic acid, and their mixtures.

The cosmetic compositions according to the invention can comprise a cosmetically acceptable vehicle that acts in the cosmetic composition as diluent, dispersant or vector of CLA in the form of free fatty acid or as triglyceride.

The amount of said vehicle can vary from approx. 50 wt.% to approx. 99.95 wt.%, preferably from approx. 80 wt.% to 99.5 wt.% of the total composition. Non-aqueous vehicles can include liquid or solid emollients, silicones and solvents.

Further cosmetic ingredients can also be incorporated in the cosmetic compositions, for example surfactants and emulsifiers for forming O/W or W/O emulsions. Other ingredients can include thickeners, preservatives, emollients, sun filters, pigments, opacifiers and perfumes.

The cosmetic compositions for skin treatment according to the invention can be formulated as solution, lotion, cream or gel, and packed in a suitable container based on the viscosity and convenience of use for the consumer. A fluid form can be packed in a bottle or a roller-ball applicator, whereas a thick cream can simply be packed in a tube or in a pot.

The following examples illustrate preferred embodiments of the invention, but are not intended to limit its scope.

5 Preparative Example I - Synthesis of CLA from grapeseed oil in glycerol

A 1-litre heated reactor, equipped with a thermometer and with a bubbler connected to a nitrogen pump, is charged with 100 g of clarified grapeseed oil,  
10 then 35 g of 85% caustic potash and 100 g of doubly-distilled glycerol.

It is heated to 220°C, keeping the mixture gently stirred. At the end, it is left to cool to 90°C, the mixture tends to assume a high viscosity, a volume of  
15 water is added, heating for about half an hour, and finally 50% sulphuric acid in several portions until there is acid reaction (pH 3) of the aqueous phase.

The oily supernatant is left to settle for 12 hours, separated, then blown with steam, filtered,  
20 washed 3 times with distilled water, anhydrous sodium sulphate is added, and it is finally filtered.

About 90 g of CLA at 70% c.a. is obtained. Chromatographic analysis of the methyl esters shows the following composition of fatty acids:

25

9c,11t- 8c,10t- octadienoic acids	30.90%
11c,13t- 10t,12c- octadienoic acids	32.05%
11t,13c- 8c,10c- 9c,11c- octadienoic acids	1.55%
10c,12c-octadienoic acid	0.65%
11c,13c-octadienoic acid	0.15%
11t,13t, 9t,11t- 10t,12t- 8t,10t- octadienoic acids	4.05%
C16 and C18 saturated and monounsaturated fatty acids	30.65%

Preparative Example II - Synthesis of CLA from sunflower oil in glycerol

The method of Preparative Example II is repeated  
30 on 100 g of sunflower seed oil, obtaining approx. 90 g of CLA at 60% c.a. Chromatography of the methyl esters revealed the following composition of fatty acids:

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9c,11t-8c,10t- octadienoic acids	26.50%
11c,13t- 10t,12c- octadienoic acids	29.40%
11t,13c- 8c,10c- 9c,11c- octadienoic acids	1.65%
10c,12c-octadienoic acid	0.65%
11c,13c-octadienoic acid	0.30%
11t,13t, 9t,11t- 10t,12t- 8t,10t- octadienoic acids	4.00%
C16 and C18 saturated and monounsaturated fatty acids	38.05%

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Preparative Example III - Synthesis of CLA from sunflower oil in diethyleneglycol

5       The method of Nichols P.L. et al. (JAOCS, 73, 247-252, 1951) was applied to 100 g of grapeseed oil, obtaining approx. 90 g of CLA at 70% c.a.

10       However, some variations were introduced, for example the reaction temperature was maintained at 220°C for 3 hours, and the product was not submitted to methylation, except for the aliquot necessary for verifying the composition of fatty acids, which was:

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9c,11t- 8c,10t- octadienoic acids	29.60%
11c,13t- 10t,12c- octadienoic acids	31.50%
11t,13c- 8c,10c- 9c,11c- octadienoic acids	1.60%
10c,12c-octadienoic acid	0.45%
11c,13c-octadienoic acid	0.25%
11t,13t, 9t,11t- 10t,12t- 8t,10t- octadienoic acids	5.10%
C16 and C18 saturated and monounsaturated fatty acids	31.50%

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15   Example IV - Synthesis of CLA from castor oil

4 g of castor oil was dissolved in 30 ml of pyridine, then 3.15 g of tosyl chloride was added, stirring at room temperature for 12 h.

20       Then 60 ml of water and 20 ml of petroleum ether were added. The organic phase was washed with 2 x 30 ml of dilute acetic acid, 3 x 30 ml of water and 10 ml of saline water, followed by vacuum evaporation, obtaining approx. 5 g of tosylated castor oil.

25       The tosylate was dissolved in 10 ml of toluene, and 3.5 g of DBU (diazabicycloundecene) was added,

heating for 5 h at 95°C. Then it was poured into 50 ml of water while stirring, and then 10 ml of petroleum ether was added, followed by washing with acetic acid, water and saline water as in the preceding case.

5 About 2.7 g of yellow-brown oil is obtained, which was found to be formed of CLA triglyceride at 87% purity, as illustrated by the following chromatographic analysis of the methyl esters of the fatty acids:

9c,11t-octadienoic acid	69.55%
9c,11c-octadienoic acid	19.51%
C16 and C18 saturated and monounsaturated fatty acids	10.94%

10

N.B. The technique described in the example is liable to numerous improvements in industrial application. The following substitutions can be mentioned as examples: a) of tosyl chloride with mesyl chloride; b) of pyridine as solvent-catalyst with DMAP (dimethylaminopyridine) or similar (for example 4-(2-imidazolidiny1)-pyridine)) in catalytic amount (1:10 equivalents) in solution of THF, toluene or other industrial solvent; c) of the double sequence of washings used, with a single final, continuous washing in appropriately arranged industrial centrifuges, etc.

15 Application Example I - Capsules of soft gelatin

20 Capsules of soft gelatin are prepared by a pharmaceutical method. Each 1.35 g capsule contains:

25

	Capsule (A)	Capsule (B)	Capsule (C)
CLA triglyceride from Preparative Example IV	-	0.8 g	0.6 g
Soya fatty acids	0.99 g	0.19 g	-
alpha-Tocopherol	0.01 g	0.01 g	0.1 g
beta-Carotene	-	-	0.05 g
alpha-Lipoic acid	-	-	0.25 g
Beeswax	0.1 g	0.1 g	0.1 g
Gelatin	0.25 g	0.25 g	0.25 g

Example I - Clinical study of the peroxidative state of the plasma for administration of CLA and the combination CLA + antioxidants

5 Six volunteers were selected, divided into 3 pairs, each of which was supplied with a pack of 60 capsules of type (A), (B) and (C), with a dosage of 3 capsules per day. The subjects were further instructed not to alter their typical style of physical activity and eating.

10 At intervals of 0, 5, 10 and 20 days since taking the capsules, the state of plasma lipoperoxidation was monitored by means of the d-ROMs kit test (Gallarati-IRAM srl, Parma, Italy).

15 Briefly, 40 µl of blood was taken by puncture of the finger at 0, 10 and 20 days of the oral treatment. Analysis was carried out immediately after obtaining the sample, the 40 µl capillary was placed in 3.92 ml of acetate buffer solution at pH 4.8 containing 40 µl of N,N-diethyl-para-phenylenediamine. After dissolution and addition of a drop of enzyme solution, the sample was centrifuged at 3000 rpm for 3 minutes, then placed in a cuvette and heated at constant temperature for 3 minutes, then the absorbance was measured at 505 nm. The results are shown in Table I.

25 TABLE I

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Comparison of lipoperoxidative level

	Mean value on day 0 (*)	Mean value on day 10 (*)	Mean value on day 20 (*)
Subjects with capsules (A)	227	235	221
Subjects with capsules (B)	216	295	342
Subjects with capsules (C)	231	246	238

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(\*) The oxidative stress is expressed as Carratelli Units (U. Carr.), where 1 U.Carr. corresponds approximately to a concentration of 0.08 mg % of hydrogen peroxide.

30



The levels of lipoperoxidation measured on the subjects treated with the combination CLA+antioxidants remain within physiological limits, in contrast to the subjects treated with CLA only.

5 Thus, it can be seen that there is a possible advantage of the method of combined treatment, which can provide, in the medium to long-term, the biological benefits typical of CLA without the exacerbations due to the peroxidative burden.

10 Example II - Lipolytic activity in vitro

The comparative lipolytic activity was measured by the methods of Chernick et al. (J. of Lipid Research, 27, 266-294, 1986), Hirsch et al. (J. of Lipid Research, 25, 665-677 (1984) and Kawamura et al. (Proc. Natl. Acad. Sci. USA, 78, 732-736, 1981).

15 Briefly, cultures of mouse embryo fibroblasts 3T3-L1 were grown in 90% DMEM with 4.5 g/l D-glucose and L-glutamine, 10% fetal calf serum and 1% of antibiotic/antimycotic liquid (100x), incubated at 37°C with 5% CO<sub>2</sub>, 95% air, then 0.5% trypsin-EDTA. They were centrifuged and then resuspended in 1 ml of 90% DMEM, 5% FCS and 10% DMSO in sterile cryogenic vials. The results are shown in Table II.

TABLE II

25

Measurement of the lipolytic effect of CLA and lipolytic substances

Substance	Concentration (g/100 ml)	% Increase relative to the control
Control	-	1.0
Isoproteronol	0.0001	7.8
CLA	0.1	1.4
CLA:caffeine		
(mix 1:1 w/w)	0.1	3.2
Caffeine	0.1	2.2

The experiments show that CLA has lower lipolytic activity than caffeine at the same concentration, but combining the two substances has a synergistic effect.

Even though CLA is less active than isoproterenol, its use is preferred on account of absence of the side effects typical of said drug.

#### Application Example II - Body cream

O/W emulsions were prepared by mixing together, with stirring, the ingredients of the oily and aqueous phase heated separately at 75°C. 100 g of each emulsion contains:

	Emulsion (A)	Emulsion (B)	Emulsion (C)
<u>Oily phase</u>			
Free CLA from Preparative Example II	-	1.7 g	1.7 g
Soya fatty acids	1.7 g	-	-
Polyglyceryl-2-sesquistearate	1.0 g	1.0 g	1.0 g
Beeswax	0.3 g	0.3 g	0.3 g
Magnesium stearate	0.5 g	0.5 g	0.5 g
Aluminium stearate	0.5 g	0.5 g	0.5 g
7-Polyoxyethylene hydrogenated castor oil	2.0 g	2.0 g	2.0 g
Low-viscosity mineral oil	10.0 g	10.0 g	10.0 g
Methyl p-hydroxybenzoate	0.1 g	0.1 g	0.1 g
18-beta-Glyceritic acid	1.0 g	1.0 g	1.0 g
Tocopheryl acetate (vitamin E acetate)	0.5 g	0.5 g	0.5 g
BHT	0.3 g	0.3 g	0.3 g
<u>Aqueous phase</u>			
Glycolic acid	3.0 g	3.0 g	3.0 g
Maté extract (caffeine 7%)	-	-	2.0 g
Decaffeinated maté extract	2.0 g	2.0 g	-
Ascorbic acid (vitamin C)	0.01 g	0.01 g	0.01 g
Deionized water qsf.	to 100 g	to 100 g	to 100 g

Example II - Clinical study of anti-cellulitic activity  
for topical application of CLA and CLA+xanthin  
combination

5 Six female subjects were first evaluated with  
respect to the extent of the cellulitic phenomenon in  
the "osterolateral" zone in the position of the lower  
limb bent to 90°, classified according to the following  
parameters.

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0	absence of cellulite
1	small skin wheals or folds
2	small wheals and streaks
3	pronounced nodules and streaks
4	all the above together with regular hard nodular bumps

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10

Six subjects were selected with degrees of  
cellulite equal to 1 or 2, who exhibited bilateral  
symmetry between the left thigh and the right thigh.

15 The volunteers were divided into 3 pairs, and each  
was supplied with cream - Emulsion A, B and C of the  
Application Example - with the instruction to apply it  
twice daily, morning and evening, exclusively on the  
area of the right thigh by gentle massaging. The  
subjects were instructed not to alter their typical  
20 style of physical activity and eating.

After application for two months, the basic  
parameters indicated by Smith W.P. (Cosmetics &  
Toiletries, 61-70, July 1995) were monitored, comparing  
the right thigh with the left thigh. The results are  
25 shown below in Table III.

TABLE III

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Comparison of anti-cellulitic properties

Subjects	Subjects	Subjects
with	with	with
Emulsion	Emulsion	Emulsion
(A)	(B)	(C)

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Thigh diameter	-1%	-5%	-8%
Thickness of the adipose layer	-3%	-18%	-24%
Subjective improvement	+10%	+33%	+50%
Clinical classification	+2%	+30%	+30%
Firmness of the skin	+5%	+10%	+15%
Irritation reactions	1	0	2
Skin hydration	+25%	+13%	+24%
Smoothness of the surface	+14%	+21%	+37%

The composition containing CLA proved effective in improving cellullitic condition, and significantly more effective in combination with 2% caffeine.

5        It has been found in practice that the present invention achieves the intended aims. Obviously the compositions and the methods according to the invention can be varied within the scope of the inventive concept.

10       The purpose of the invention is better defined by the claims, rather than by the examples given above.

**CLAIMS**

1. Method of preparation of conjugated linoleic acid (CLA), characterized in that the reaction is carried  
5 out with the reactants (a), (b) and (c), where:  
(a) is a vegetable oil with content of linoleic acid not less than 60%;  
(b) is glycerol;  
(c) is an alkaline hydroxide  
10 said method being carried out at a temperature between 200 and 240°C for 2-8 hours, followed by neutralization with aqueous mineral acids, separation by decanting or by centrifugation of the oily phase, and recovery of said CLA, optionally submitted to filtration, drying,  
15 deodorizing or clarification.
2. Method of preparation of CLA according to claim 1, in which reactant (a) is selected from grapeseed oil, Carthamus oil, sunflower oil, corn oil, and their mixtures.
- 20 3. Method of preparation of CLA according to claim 1, in which the weight ratio of glycerol (b) to vegetable oil (a) is between 0.5:1 and 4:1.
4. Method of preparation of CLA according to claim 2, in which the alkaline hydroxide (c) is caustic soda,  
25 caustic potash, or mixtures thereof, in stoichiometric ratio from 1:1 to 5:1 relative to the vegetable oil (a).
5. CLA in the form of free acid or its soap (metal salt) obtained by a method according to claims 1-4.
- 30 6. CLA in the form of free acid or its soap (metal salt) obtained from grapeseed oil by alkaline isomerization.
7. Method of preparation of CLA triglyceride in which castor oil having the 12-hydroxyoleic residues bound to  
35 an aryl- or alkyl-sulphonic group is treated with tertiary amines.
8. Method according to claim 7, in which the sulphonic group is tosylate, mesylate or triflate.

9. Method according to claim 8, in which the tertiary amines are selected from the group comprising pyridine, diazabicycloundecene, 4-N,N-dimethylaminopyridine, 4-(2-imidazolidinyl)-pyridine, and their mixtures.

5 10. CLA in the form of triglyceride obtained by a method according to claims 7-9.

11. Method of improving the state of physical well-being by oral administration of a dietetic preparation containing the following components:

- 10 a) CLA in the form of free acid or triglyceride, in amounts between 0.01 and 50 wt.% of the preparation.  
b) Dietetically acceptable antioxidants, with weight ratio of antioxidant to CLA between 1:2 and 5:1.  
c) Dietetically acceptable nutrients and excipients,  
15 for the remainder of the preparation.

12. Dietetic preparation according to claim 11, where the antioxidants (c) are nutritional substances contained in seeds, leaves, flowers and in the cortex of fruit and vegetables, extracted from natural sources  
20 or the equivalents of synthetic origin.

13. Dietetic preparation according to claims 11 and 12, where the nutritional antioxidant substances are selected from the group comprising histidine, valine, aromadendrene, morin, quercetin, hesperetin,  
25 naringenin, kaempferol, apigenin, luteolin, fisetin, fustin, rutin, myricetin, pelargonidin, cyanidin, delphinidin, catechol, epicatechol, gallocatechol, proanthocyanosides, isomers of carotene, lycopene, zeaxanthin, retinol, cathaxanthin, alpha-, beta- and  
30 gamma-tocopherol, gallic acid, propyl gallate and gallic esters, tannic acid, egg and soya lecithin, L-ascorbic acid and esters, lactic acid, citric acid, L-tartaric acid, polyketides of Monascus red, cardanol, cardol, anacardic acid, oryzanol, anacardic oil,  
35 thiodipropionic acid and laurylic ester (DLTBP), alpha-lipoic acid, nordihydroguaiaretic acid (NDGA), guaiacol, hydroxytyrosol, oleuropeine, verbascoside), phenols from rosemary, cloves, peppers, cinnamon, ginger, paprika (TBHQ), citrus oils,

trihydroxybutyrophenol (THBP), BHT and BHA, maclurin, ethoxyquine, ellagic acid, musizine, boldine, and their corresponding glycosides, esters and ethers, and their combinations.

- 5 14. Dietetic preparation according to claims 11-13 in the form of capsule, tablet, alcoholic solution, emulsion, baby-food, milk-dairy product, sports supplement, or other formulation intended for human nutrition.
- 10 15. Method of treatment of cellulite by topical application of CLA in the form of free acid, of its metal salt or triglyceride, at a concentration from 60 to 90 wt.% of the total of the fatty acids.
- 15 16. Method of treatment of cellulite by topical application of a cosmetic composition containing CLA in the form of free acid, of its metal salt or triglyceride.
- 20 17. Cosmetic composition according to claim 16 in the form of a cream, gel, lotion, or spray, said composition containing CLA in amounts from 0.5 to 90 wt.%, and one or more cosmetically acceptable ingredients from 10 to 99.5 wt.%.
- 25 18. Cosmetic composition according to claim 17 further containing one or more antioxidant substance for cosmetic use.
19. Cosmetic composition according to claims 16 and 17 further containing one or more vegetable substance with anti-cellulitic activity.
- 30 20. Cosmetic composition according to claim 19 where said vegetable substance with anti-cellulitic activity is selected from the group comprising extracts of *Centella asiatica*, of *Hibiscus abelmoschus*, of *Polygala tennifolia*, of *Platycodon grandiflorum*, of *Kochia scoparia*, of ivy, inositol phosphate, phytic acid, ginsenosides, aescine, caffeine, theophylline, or
- 35 mixtures thereof.